The Investigation of Gene Expression in FIBP, GATA-2, SNP136, SNP hOGGI in human prostate cancer

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Introduction:

ET-1 (endothelial-1), a peptide hormone, is a potent vasoconstrictor that contributes to the increased expression of oncogenes (1). ET-1 shows increased expression in normal male human ejaculate and has a strong association with metastatic prostate cancer. It has been shown that ET-1 directly induces prostate cancer and increases the mitogenic effects of insulin-like growth factor I, insulin-like growth factor II, platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor in serum-free conditions in vitro. ET-1 expression is increased under the environment of ischemia and hypoxia since there is a hypoxia-inducible factor-1 (HIF-1) binding site in the human ET-1 promotor (3). The HIF-1 binding site contributes to the activation of ET-1 expression in endothelial cells. The conditions of hypoxia are not solely contributed to HIF-1 but 50 base pairs of flanking sequence for the binding sites of activator protein (AP-1), proliferation factor (GATA-2), and CAAT-binding factor (NF-1). Previous experimentation shows mutations at the HIF-1 site eliminated hypoxia induction but mutations at GATA-2 caused the over expression of GATA-2. GATA-2 must be studied in other models to see if it will increase the expression of oncogenes in tumor proliferation (6).

I'm studying to see if GATA-2 will act as a proliferation factor in a prostate cancer model. LNCAP is a known prostate cancer cell lie that is chosen for its androgen dependent behavior. In a biochemical setting the question is asked if the decreased expression of GATA-2 affect the growth rate of LNCAP cells (2). To find the answer to this question small interference RNA (siRNA) sequence targeted specifically to GATA-2 was used to decrease the expression GATA-2 in LNCAP cells. siRNA was introduced

into LNCAP cells in lipofectamine transfection. The authenticity of the siRNA as a mechanism for RNA interference to decrease GATA-2 expression was verified with the use of Western Blotting and Real-time PCR (RT-PCR) analysis. After the verification of the siRNA function, LNCAP cells were transfected with the siRNA. Later LNCAP cell counts were taken with siRNA and without siRNA. LNCAP cell counts increased in control groups as compared to those transfected, which demonstrates GATA-2 as a proliferation factor for LNCAP cells. Further experiments should find where in the cell cycle GATA-2 affects proliferation factors and *in vivo* murine studies should later be examined. In previous studies GATA-2 is a proliferation in cancer models that have androgen dependent behavior. If GATA-2 can be proven as a proliferation factor in LNCAP, this will give greater understanding for its role in cancer models.

In a clinical prostate cancer research setting, I'm working to gather data for possible SNP (single nucleotide polymorphism) of up-regulated FGF (fibroblast growth factor) genes. SNP is a change in one nucleotide from an exon sequence, which can lead to translation of a different protein (5). It has been shown in previous studies there is increased occurrence of SNP in cancerous tissue as compared to noncancerous tissue. The SNP study was initially based upon microarray data to show possible genes of high interest. These genes under my study were hOGGI and 136. The SNP for the change of the nucleotide G to a C leads to a formation of serine for threonine (4). The genotyping of 136 and hOGGI shows there is a statistical significance in SNP occurrence for just 136. The possible roles that 136 plays must be looked upon for further prostate cancer studies. From previous literature, 136 has a universal role as a SNP in other cancerous diseases

such as esophageal cancer (7). Since the control SNP of hOGGI is already known we can just compare the cancer SNP to the literature SNP counts.

Prostate cancer is one of the leading causes of death of men. Fibroblast growth factor (FGF) signaling is important as it is expressed at increased levels in men with prostate cancer (4). The expression of FGF can play a role in the functions of cell motility, proliferation, and the increase in tumor angiogenesis. Intracellular acidic fibroblast growth factor binding protein (FIBP) is believed to have a strong association with acidic FGF (aFGF) to activate a multitude level of responses (6). Acidic FGF has both an extracellular and intracellular function. The extracellular behavior involves it binding to fibroblast growth factor receptors (FGFRs) on the cell membrane. There are four transmembrane tryosine-kinase receptors that potentially play a role in prostate tumor progression. The binding of aFGF to FGFRs leads to the cross linking of the receptors to activate tyrosine-kinase domain, which can activate several intracellular phosphorylation cascades (1). The role of FIBP remains unclear as it moves possibly from the nucleus to the cytoplasm. It is postulated that FIBP is not involved in the transport aFGF into the nucleus but rather it binds to aFGF to activate DNA synthesis. The binding of FIBP to aFGF appears to be electrostatic in nature and the binding seems to be inhibited in the presence of heparin. The expression of FIBP appears to play an essential role in the aftermath of it binding to aFGF in the nucleus region (6). Because FGF signaling is essential in prostate cancer, I investigated the expression of FIBP in human prostate benign tissues and human prostate cancer tissues to ascertain whether there is differential expression of FIBP in human prostate cancer tissues. Previous studies show FGF being up-regulated in the prostate cancer lines versus normal cell lines. Since

FGF is established as a possible for the tumor proliferation in prostate cancer to understand the role binding factors such as FIBP play with FGF will further elucidate the complete mechanism of FGF.

Materials and Methods:

hOGGI and 136 Genotyping: To determine the distribution of SNP for hOGGI and 136 in prostate cancer patients and in control groups, the following primers were used: 5'-CCTCTTGGACCTTAGATGCTTC-3' and 5'-CTGGACAGCGGAACTTGAC-3' (137F/137R) and 5'- GGGGATTATTTTATTTTCTATG-3' and 5'-AGCAGAGGGAGAGCTTCTG-3' (hOGGIF/hOGGIR). The PCR product from a 1.5% TAE DNA gel of the primers is 317 bp in length for 136 and 250 bp in length for hOGGI. The annealing temperature that was used to generate the PCR products was 68.5C following the restriction enzyme digest. The C to G change in one nucleotide leads to change in protein of serine to threonine. A restriction enzyme digest at 37C for 4 hours of the PCR products was performed with EcoNI for 137 and FNUHI for hOGGI. This verified the presence of our SNP under analysis.

Construction of FIBP plasmid standard: Oligonucleotide primers were designed as FIBPC25 forward (5'-ACCAGTGAGCTGGACATCTTCG-3') and FIBPCR15 reverse (5'-GGGAGTGGACAGACGCTGAAT-3') to amplify a 987 bp fragment encompassing the FIBP cDNA in a PCR reaction. A standard PCR reaction was carried out using LNCAP cDNA as template in a reaction containing 0.4μM of the forward and reverse primers and the following conditions (initial denaturing at 95^{o}C for 3 minute, followed by 34 cycles of denaturing at 95^{o}C for 30 seconds, annealing at 60^{o}C

for 30 seconds and extension at 70°C for 1 minute and a final extension at 72°C for 7 minutes). The PCR reaction was analyzed by gel electrophoresis on 1% agarose gel and the 987 bp fragment purified from the gel using Qiagen gel purification kit (and according to the manufacturer's protocol). The 987 bp cDNA fragment was subsequently cloned into Topo Vector (Invitrogen; and according to the manufacturer's protocol) and the recombinant plasmid DNA was prepared using a Qiagen kit and verified by EcoRI restriction enzyme digestion.

Quantification of plasmid was performed spectrophotometrically. The measurements of the plasmid concentration were done in duplicate and then converted to copy number (N).

N (copy number per µl) = moles X Avogadro constant.

A dilution series of each plasmid (10^9 to 10^1 copies) was used for DNA standard.

Gradient PCR is used for three sets of primers which encompassed the on 987 bp region of FIBPC25/FIBCR15 to obtain the primer of least background of non-specific binding at the range of temperatures from 50°C -72°C. Primer FIBP 1R/1F which encompasses a region of 152 bp of the FIBP gene was chosen at the annealing temperature of 68 °C.

Quantitative Real-time PCR: Quantitative Real-Time PCR (RT-PCR) was performed on a reaction using 5 µl of template cDNA in a total 25 µl reaction volume consisting of FIBP forward primer ((5'-GACCATTACCGCACCTTCC-3') and reverse primer (CAGCACCTCCCGAACAAAG) 0.4 µM of each primer, 3mM MgCl2, 2.5 µl of LC-FastStart DNA Master Sybr Green I (490 nm). RT-PCR was conducted using the iCycler instrument (Bio-Rad) with the optimal PCR conditions for FIBP amplification using the following: 3 minute hot start at 95 °C for 30s, annealing at 68 °C for 20s, and a 72°C extension for 30s. Each of the reactions consisted of a negative control, and the

experiment was done in duplicate. The fluorescent was given off by the reporter dye (Sybr Green I), and the threshold cycle (Ct) of each sample was recorded as a quantitative measure of the amount of PCR product of the sample. Then the FIBP copy number was normalized against β-actin expression levels in each of the prostate cell lines. N = (RT-PCR/β-actin N) for each of the select prostate cell lines.

Results:

Table 1 (The Real-Time expression of GATA-2 siRNA function on LNCAP cells)

Control (normal)	**siRNA (transfected)**
1. 24hr normal (10 ul H2O) expression=8.78E5 B-actin=9.9E6 **final expression=8.86E-2**	1. 24hr (20ul) 200 picmol expression=2.44E6 B-actin=4.93E7 **final expression=4.95E-2**
2. 48hr normal (10 ul H2O) expression=1.19E6 B-actin=2.4E7 **final expression=9E-2**	2. 48 hr (10 ul) 100 picmol expression=1.44E6 B-actin=1.6e7 **final expression=4.9e-2**
3. 72 hr normal (20 ul H2O) expression= 1.23E6 B-actin=1.27E7 **final expression=9.66E-2**	3. 48 hr (20 ul) 200 pic mol expression=9.07E5 B-actin=3.24E7 **final expression=2.799E-2** 4. 72 hr (10 ul) 100 picmol expression=5.06E5 B-actin=4.79E6 **final expression=1.05E-1**
	5. 72 hr (20ul) 200 picmol expression=3.95E5 B-actin=1.65E7 **final expression=2.393E-2**

It can be seen at 200 picomol or 20 ul of the siRNA transfection agent there seems to be a gradual knockdown of the GATA-2 gene with siRNA. This is going from 24, 48, and 72 hours from the control to the transfected cell line.

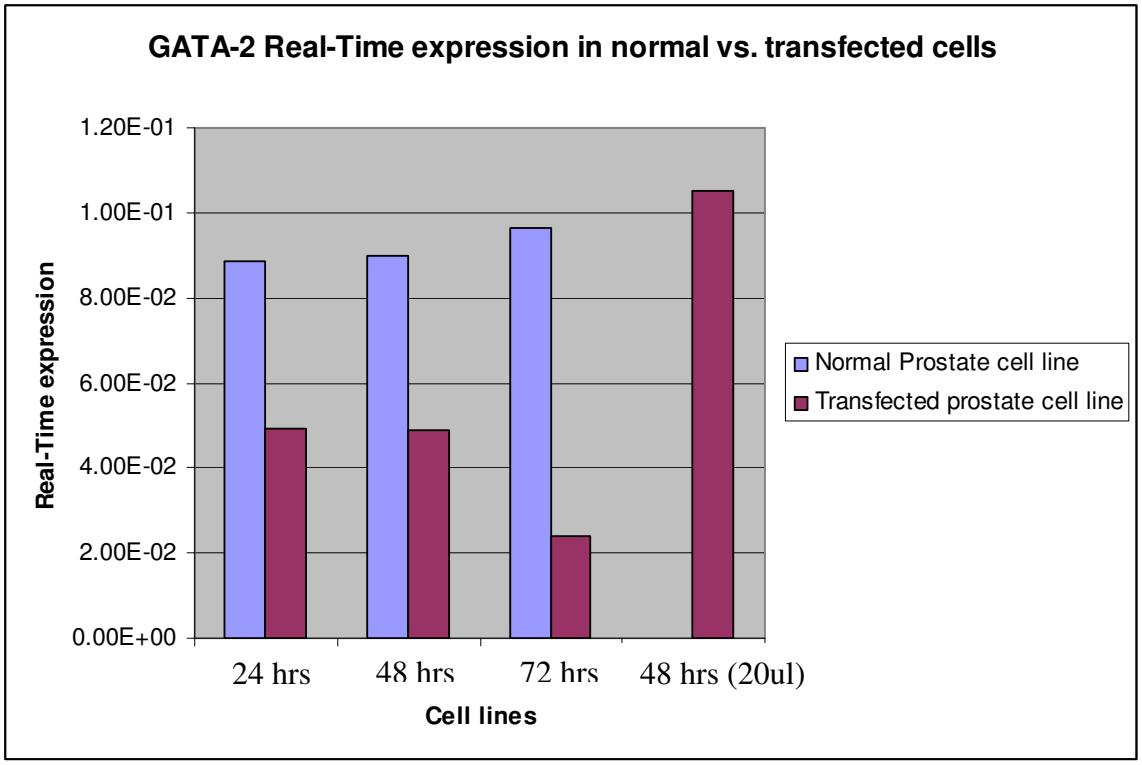

Figure 1.1: Shows the graphical representation of Table 1.1. It can be seen that the transfected cells had decreased expression of GATA-2 as compared to the control cell line over the various time periods.

Table 2 (original GATA-2 LNCAP cell counts)

SiRNA Transfection	Control (H2O)
Day 1	**Day 1**
316680	3773250
298920	363160
320040	369020
311880 **Average**	**369833** **Average**
Day 3	**Day 3**
289320	409060
293400	397400
295520	396320
292747 **Average**	**400927** **Average**
Day 5	**Day 5**
270520	420640
275610	415160

269790	421910
271973 **Average**	**419237** **Average**

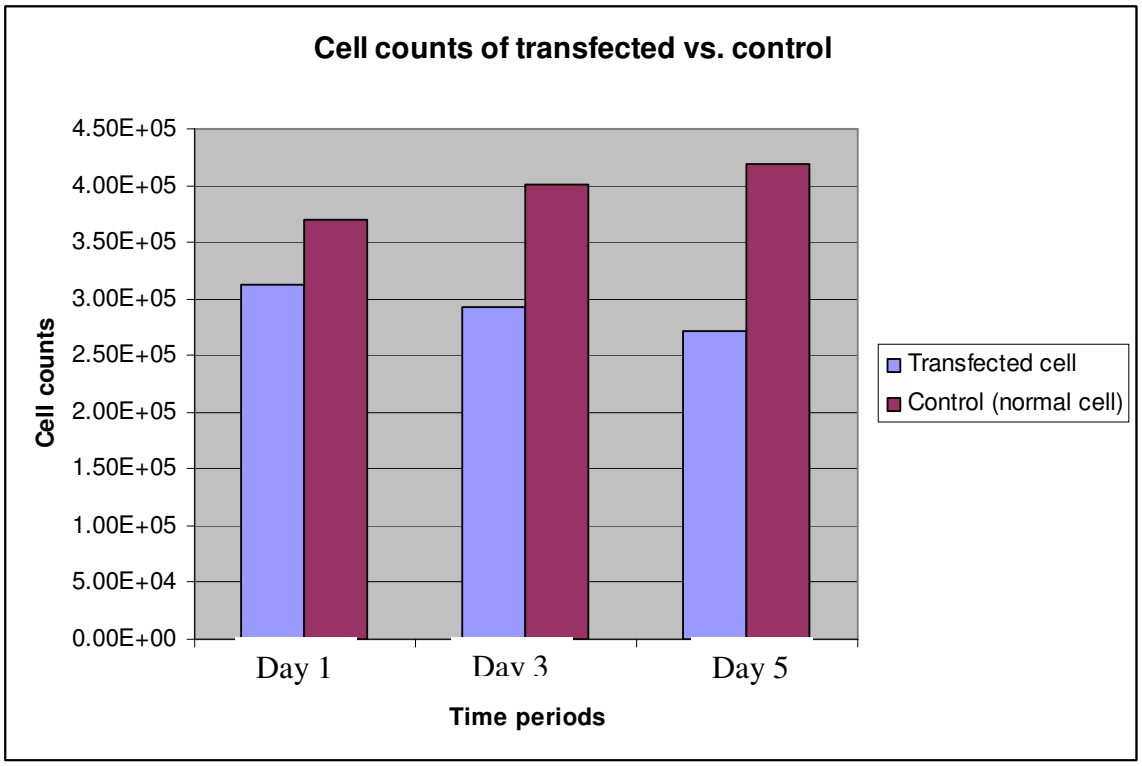

Figure 1.2: Shows a graphical representation of Table 2. It can be seen that as the time period increases there is a decrease in the cell counts from the control to the transfected cells.

Table 3 (repeat of GATA-2 LNCAP cell counts)

SiRNA transfection	**Control (H2O)**
Day 1	**Day 1**
399480	480560
381560	450050
395320	448200
392120 **Average**	**459603** **Average**
Day 3	**Day 3**
367120	663880
402800	599440
371000	602500
380307 **Average**	**621940** **Average**
Day 5	**Day 5**

226760	516000
269240	507400
269920	523080
255307 **Average**	**515493** **Average**

Both tables 2 and 3 show the GATA-2 LNCAP cells counts for both an original and a repeat cell count that is done in an experimental duplication for one, three, and five days. The experimental repeat or duplication was done out of necessity so that we could verify the results of the experiment without the possibility of experimental error. 200 picomole of the siRNA reagant was added to each 35 mm dish.

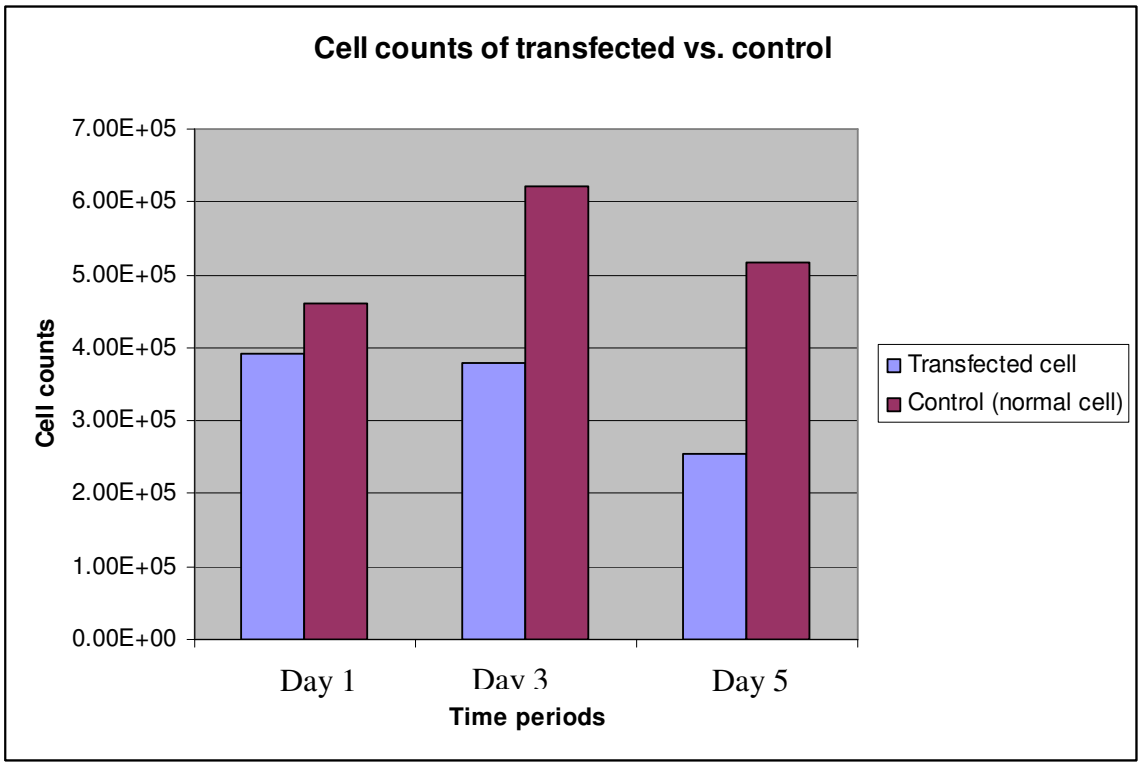

Figure 1.3: Is a graphical representation of Table 3. It can be seen the cell counts from the transfected to the control cells do decrease. Yet, there is concern for the Day 5 cell counts since the control and the transfected cells counts both decrease. Part of the decrease is attributed to competition between the cells and limited availability of resources after a long period of time.

Table 4 (shows the SNP for 100 human cancer samples for hOGGI using the restriction enzyme FNUHI)

Cancer sample	Genotype	Cancer sample	Genotype
4895	CG	37374	CC
21450	GG	17801	CC
34941	CC	21131	CG
25417	CG	7881	CC
10992	CG	4853	CC
8907	CG	10851	CC
9364	CC	3330	CC
5405	CC	10968	CC
7977	CC	37374	CC
7909	CC	17801	CC
9635	GG	21131	CG
1950	CC	7881	CC
17780	CC	4853	CC
15238	CC	10831	CC
2167	CG	3330	CC
4002	CG	19553	CG
13604	CC	36363	CC
2464	CG	18797	CC
11069	CG	3779	CC
10891	CC	4590	CG
20163	CC	5936	CG
10684	CC	19439	CG
32043	CC	8712	GG
25588	CC	8172	GG
17780	CC	9055	CC
9862	CG	37018	CC
7731	CC	4514	CC
18137	CC	19979	CC
9580	CG	9528	CC
19250	CC	12404	CC
3124	CC	17480	CG
1940	CG	7484	CC
3490	CC	19010	CC
21627	CC	18942	CC
20274	CC	20945	CG
18990	CC	11403	CC
8474	CC	10770	CC
21014	CC	11155	CC
21382	CC	10060	CC

2800	CG	8240	CG
26156	CC	7234	CG
9772	CC	19019	CC
19010	CC	15258	CC
18942	CC	22328	CC
20945	CG	21062	CC
11043	CC	4867	CG
32458	CC	4709	CG
19987	CC	17575	CG
6506	CG	10205	CC
9019	CC	1791	CC

Sample	**Relative FIBP expression**
Normal	1.26E-03
BPH	1.38E-03
Cancer	1.77E-03

Table 5: To investigate the expression of FIBP in benign and cancerous tissues we used RT-PCR. We analyzed cDNA consisting of 20 tissues of high recurrence, 20 tissues of low recurrence, 19 tissues of non-recurrence, 8 tissues of benign prostate hyperplasia (BPH), and 14 normal prostate tissues. Results of RT-PCR data expressed relative to β-actin.

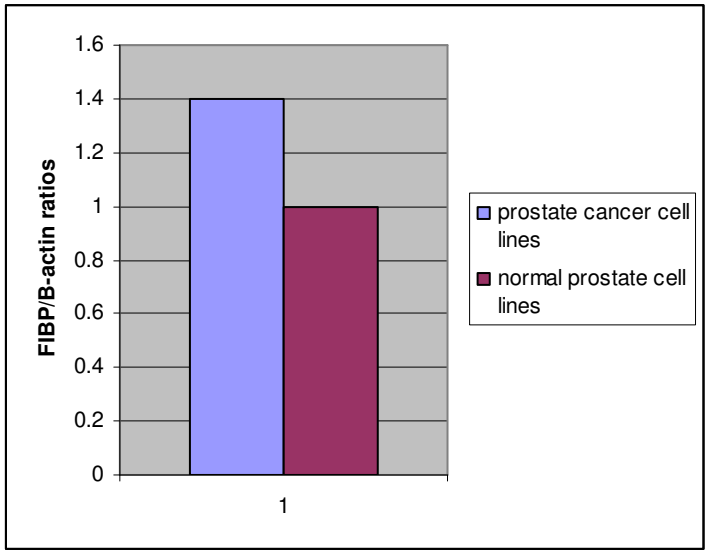

Fig. 1.4: Comparative expression of FIBP amongst cancer and normal prostate cells using quantitative RT-PCR. FIBP expression was normalized using β-actin with the ratio (FIBP N/β-actin N). Overall relative expression ratios are seen.

Discussion:

The Real-Time PCR data in Table 1 shows the various siRNA transfection amounts given and demonstrates 200 picomole of siRNA as the optimal reagent needed to perform the cell counts on. Results from Table 1 are needed since it verifies the optimal condition to use GATA-2 in a lipovectamine transfection to assess its possible role as a proliferation factor in LNCAP cells. LNCAP cell counts taken for GATA-2 show decreased cell counts when siRNA was administered. The siRNA lipovectamine transfection decreased the expression of GATA-2 in Table 1 for the Real-Time PCR experiment and decreased LNCAP cell counts. The LNCAP cells with siRNA transfection reagent were compared to control LNCAP cell counts without siRNA reagent. The decreased LNCAP cell counts with siRNA reagent prove that GATA-2 acts as a proliferation factor for LNCAP cells. With the importance of GATA-2 as a mechanism for the proliferation of the LNCAP cells there comes to question the functional role that GATA-2 can play for proliferation. To understand at a greater depth the mechanistic approach that GATA-2 plays, one should perform a cell cycle assay to determine in which part of the cell cycle that GATA-2 plays an important role. This will give greater insight into the precise steps that GATA-2 plays in the proliferation of LNCAP cells. Moreover, since it is seen that GATA-2 plays a mechanistic role for increased expression we should try to find a better means for its stepwise methodology. Furthermore, other experiments must be designed to obtain the mechanistic role of GATA-2 as a proliferation factor. Future experiments are now underway to use flow cytometry to understand where in the cell cycle GATA-2 increases its expression to proliferate prostate cancer cell counts. The biology of GATA-2 can be investigated using

a murine model to test and understand the exact effects that GATA-2 when there is decreased and increased expression. By having a model organism to represent the expression profile that GATA-2 plays one can determine its role of regulation within the cell as a whole.

In the investigation of hOGGI, the genotype frequencies were generated from the 100 human cancer samples; the allele frequencies came out to be 69% CC (Ser/Ser), 27% CG (Ser/Cys), 4% GG (Cys/Cys). This data for cancer SNP of hOGGI is in a strong correlation to the published data for control of American (Caucasian) males, which were 75.2% CC (Ser/Ser), 23% CG (Ser/Cys), 4% GG (Cys/Cys). This data shows there is not enough significance to claim that there is difference for the allele frequencies of hOGGI SNP to occur. The future studies of the hOGGI SNP can still be studied in other cancer models to assess if there is difference in the data from the cancer to the control allele frequencies.

The increased expression of FIBP in cancerous tissues suggests that FIBP could play a possible role in the up regulation of human prostate tumor angiogenesis. There is a 40% increase in the expression of FIBP in cancer as compared to the normal prostate cancer cell line. The expression of FIBP in the prostate cancer cell expression is significant enough to determine its importance in the role of the tumor proliferation.
Due to aFGF intracellular nature, further studies need to be conducted to see the expression levels of FIBP in response to aFGF stimulation of normal prostate and prostate cancer cells in vitro and also the biological effects of over-expression/down-regulation of FIBP in prostate cancer cells.

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